



# The Role of *ex-vivo* Gene Therapy of Vein Grafts with Egr-1 Decoy in the Suppression of Intimal Hyperplasia

M. Peroulis<sup>a,b</sup>, J. Kakisis<sup>a,b,\*</sup>, A. Kapelouzou<sup>a</sup>, A. Giagini<sup>a</sup>, S. Giaglis<sup>a</sup>, G. Mantziaras<sup>a</sup>, N. Kostomitsopoulos<sup>a</sup>, P. Karayannacos<sup>a</sup>, A. Macheras<sup>b</sup>

<sup>a</sup> Center of Experimental Surgery, Foundation of Biomedical Research, Academy of Athens, Athens, Greece

<sup>b</sup> 3rd Department of Surgery, Athens University Medical School, 'Attikon' Hospital, Athens, Greece

Submitted 16 September 2009; accepted 28 April 2010

Available online 7 June 2010

## KEYWORDS

Egr-1;  
Decoy;  
Gene therapy;  
Graft occlusion;  
Vascular;  
Intimal hyperplasia;  
Endothelial cells

**Abstract** *Objectives:* To test the hypothesis that vein graft intimal hyperplasia can be significantly suppressed by a single intra-operative transfection of the graft with a decoy oligonucleotide (ODN) binding the transcription factor Egr-1.

*Design:* Experimental study.

*Materials and methods:* Jugular vein to carotid artery interposition grafts in rabbits were treated with Egr-1 decoy, mutant decoy ODN, vehicle alone, using a non-distending pressure of 300 mmHg for 20 min, or were left untreated. All animals were fed a 2% cholesterol diet. The animals were sacrificed after 48 h, 6 weeks and 12 weeks. Paraffin-embedded vein sections were subjected to angiometric analysis.

*Results:* Successful delivery of the ODN was confirmed by DAPI staining. Quantitative real-time PCR revealed a 60% decrease of the Egr-1 gene expression in the animals in which the Egr-1 decoy ODN was delivered. Cellular proliferation was also significantly decreased as indicated by the Ki-67 labelling index. An increase in intimal and medial thickness was found in all vein grafts. However, intimal thickness was significantly reduced in the grafts treated with Egr-1 decoy ODN, whereas luminal area was significantly increased.

*Conclusion:* A single intra-operative pressure-mediated transfection of vein grafts with Egr-1 decoy ODN significantly suppresses intimal hyperplasia in a rabbit hypercholesterolaemic model.

© 2010 European Society for Vascular Surgery. Published by Elsevier Ltd. All rights reserved.

\* Corresponding author at: 23 El. Venizelou Street, New Psychico, Athens 15451, Greece. Tel.: +30 210 6754474; fax: +30 210 6728538.  
E-mail address: [kakisis@med.uoa.gr](mailto:kakisis@med.uoa.gr) (J. Kakisis).

More than a century after their initial description by Carrel and Guthrie in 1906, vein grafts remain the best arterial substitute for peripheral vascular reconstruction, despite the significant progress in the technology of synthetic bypass grafts.<sup>1,2</sup> The main cause of failure of vein grafts is the development of intimal hyperplasia.<sup>3,4</sup> Although several approaches, such as pharmacologic agents, external sheaths and stents, external radiation, brachytherapy or photodynamic therapy, have been tried for the prevention of intimal hyperplasia, none of them has solved the problem.<sup>5–10</sup> Recent advances in the understanding of the intracellular molecular mechanisms mediating the development of intimal hyperplasia have provided novel targets for preventive and therapeutic interventions.<sup>11–13</sup>

Egr-1 (early growth response factor-1) is a zinc finger transcription factor, first identified by Sukhatme and colleagues in 1988.<sup>14</sup> It is expressed in several cell types in the vascular wall, including endothelial cells (ECs), vascular smooth muscle cells (SMCs), monocytes and macrophages. Egr-1 expression is induced by multiple stimuli associated with the development of vascular diseases, such as shear stress, mechanical stress, hypoxia, insulin and glucose, angiotensin II and acute arterial injury.<sup>15–17</sup> Once activated, Egr-1 induces the expression of multiple pro-inflammatory genes involved in atherosclerosis, such as monocyte chemoattractant protein-1 (MCP-1), tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) and intercellular adhesion molecule-1 (ICAM-1), as well as the expression of genes, such as fibroblast growth factor-2 (FGF-2), platelet-derived growth factor (PDGF) and transforming growth factor- $\beta$  (TGF- $\beta$ ), implicated in the proliferative and chemotactic response to injury leading to intimal hyperplasia and restenosis.<sup>15–17</sup>

The aim of our study was to test the hypothesis that a single intra-operative pressure-mediated transfection of jugular vein to carotid artery interposition grafts with Egr-1 decoy oligonucleotide (ODN) would significantly suppress intimal hyperplasia in a rabbit hypercholesterolaemic model.

## Materials and Methods

### Oligonucleotides

A double-stranded Egr-1 decoy phosphorothioate-stabilised ODN, (Invitrogen Corporation, Carlsbad, CA, USA) which contains two copies of 5'-GCGGGGCG-3' representing the Egr-1 consensus binding sequences, was synthesised.<sup>18</sup> The mutant Egr-1 decoy ODN used as a negative control contained the following sequence: 5'-GCTAGGGCG-3'.<sup>18</sup> The ODNs were dissolved at a concentration of 40  $\mu\text{mol l}^{-1}$  in normal saline solution. Fluorescein isothiocyanate-labelled Egr-1 decoy ODN (FITC-ODN) was used for fluorescent microscopic evaluation of ODN distribution after transfection.

### Experimental animal model and ex-vivo transfection

Sixty-six male New Zealand white rabbits (*Oryctolagus cuniculus*) 12–15 weeks old, weighing 3–3.5 kg were used in this experiment. The experimental protocol was approved by the ethics committee of the Foundation of

Biomedical Research. The animals were housed individually and cared for in accordance with the guiding principles of the American Physiological Society and the Greek Presidential Decree 160/1991, issued after the European Union Directive 609/1986.

All animals were fed a diet containing 2% cholesterol starting 1 week before the operation until the time of vein graft harvest. At operation, animals were premedicated with intramuscular administration of xylazine (2–5  $\text{mg kg}^{-1}$ ) and ketamine (25–50  $\text{mg kg}^{-1}$ ) and were mechanically ventilated after tracheal intubation (VMS, MDS Matrix, USA). Anaesthesia was maintained with the administration of iso-flurane (2.05% Forenum, Abbott, Abbot Park, IL, USA). The auricular vein was catheterised for fluid administration. Heart rate, respiratory rate,  $\text{SpO}_2$ , electrocardiograph (ECG), rectal temperature, systolic, diastolic and mean arterial pressure were continuously monitored (Passport 2, Datascope, USA). A midline vertical neck incision was performed and a 2.5-cm segment of the right external jugular vein was harvested using a non-touch technique. The vein graft was cannulated distally with a 22-G vein catheter and secured with a 3/0 silk tie. The proximal end remained open and the graft was flushed gently with normal saline solution. The graft was surrounded by a plastic sheath, which was mounted proximally on the vein catheter with a 2 silk suture. The ODN solution (decoy, mutant decoy or fluorescent isothiocyanate (FITC)) or vehicle alone was infused through the cannula so that both the lumen of the vein and the surrounding space were bathed in the fluid. The plastic sheath was clamped distally and the fluid was pressurised to 300 mmHg with the use of a balloon inflation device (Styker Diskmonitor).

Heparin (200 U  $\text{kg}^{-1}$ ) was administered intravenously and the ipsilateral common carotid artery was clamped proximally and distally and divided. The vein graft was anastomosed to the divided artery in a reversed end-to-end fashion with interrupted 7/0 polypropylene sutures. After removal of the arterial clamps, the proximal and distal ends of the common carotid artery were treated with lidocaine 2% and the wound was closed. All animals recovered in the Intensive Care Unit under continuous monitoring.

Animals were divided in five groups. Vein grafts in group A (12 animals) received treatment with Egr-1 decoy solution (40  $\mu\text{mol l}^{-1}$ ); in group B (18 animals) they were treated with mutant decoy ODN (40  $\mu\text{mol l}^{-1}$ ); while in group C (12 animals), vein grafts were treated with vehicle alone. Vein grafts in group D (18 animals) received no treatment, while grafts in group E (six animals) were treated with FITC-labelled decoy (FITC-ODN). Half of the animals in groups A and C were sacrificed after 6 weeks and the other half after 12 weeks. One-third of the animals in groups B and D were sacrificed after 48 h, one-third after 6 weeks and the other third after 12 weeks. Grafts excised after 6 and 12 weeks were carefully dissected to remove adherent tissue, perfusion fixed *in situ* at 80 mmHg, and harvested and processed for routine paraffin embedding. Grafts excised after 48 h were homogenised and subjected to quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) to evaluate Egr-1 expression.

Animals in group E were sacrificed after 48 h, following verification of their patency by colour Duplex scan. The vein grafts were removed and divided in two segments. One

of these was paraffin embedded and subjected to nuclear staining with 4',6-diamidino-2-phenylindole (DAPI) (Roche Molecular Biochemicals, Indianapolis, IN, USA) to measure transfection efficiency, whereas the other half was homogenised and analysed with the use of RT-PCR to evaluate Egr-1 expression.

### Vessel morphometry

Extents of 5- $\mu$ m cross sections from the middle of the vein grafts as well as from the contralateral external jugular vein were treated with haematoxylin–eosin and sirius red for collagen staining and with Orcein for elastin staining. All measurements were conducted using image-processing software (Image-Pro Plus v4.5; Media Cybernetics Inc, Silver Spring, MD, USA) on polychromatic images taken by a digital camera (Altra20; Soft Imaging System, Munster, Germany) coupled to light microscopy (Olympus CX31; Olympus, Tokyo, Japan). Thickness and area were determined under suitable magnification from haematoxylin and eosin-stained sections. Measurements of wall thickness and of intimal and medial thickness were performed in 20 representative optical fields every 18° around the vessel circumference in appropriately stained sections. For each animal, three cross sections were measured for calculating mean values. Neointima was distinguished by its position relative to the internal elastic lamina and by its chaotic organisation of cells compared with the circumferential arrangement of medial SMCs. All measurements in each sample were made by two independent observers blinded to treatment allocation.

### Reverse transcription quantitative real time PCR

Real-time RT-PCR was performed to confirm Egr-1 mRNA expression in vein grafts from *O. cuniculus*. Total RNA was isolated using TriReagent (Sigma) with DNase treatment (RNase-Free DNase Kit, Qiagen). Reverse transcription was performed using MMLuV RT (Promega) and random primers. PCR primers were designed using Beacon Designer 7.0 software (Premier Biosoft, Palo Alto, CA, USA). The primers for *O. cuniculus* Egr-1 were as follows: forward: 5'-CCCGAC-TACCTGTTTCCG-3'; reverse: 5'-GTGGAGAGTGGAGTGAGG-3'. The primers for  $\beta$ -actin were as follows: forward: 5'-TGTCCTGTACGCCTCTG-3', reverse: 5'-CGTAGCCCTCG-TAGATGG-3'. The primers were tested for specificity by conventional PCR. Reaction conditions were optimised, and cDNA samples were subjected to PCR amplification using SybrGreenER PCR SuperMix (Invitrogen Corporation, Carlsbad, CA, USA) using the Chromo4 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Values were determined using Opticon Monitor 3 software (Bio-Rad) and normalised to  $\beta$ -actin. The  $2^{-\Delta\Delta C_t}$  method was used for quantification of the target gene.<sup>19</sup>

### Immunohistochemical staining technique for Ki-67

Serial embedded paraffin sections of vein grafts were incubated with an anti-mouse Ki-67 monoclonal antibody (DAKO, Glostrup, Denmark) at a dilution of 1:80 for 90 min at 37 °C to identify cell proliferation. Antibody binding was

visualised using the labelled streptavidin–biotin (LSAB) method (DAKO LSAB Kit), and 3,3-diaminobenzidine. Then, cell nuclei were counterstained with haematoxylin and mounted with DPX. The percentage of Ki-67 positive cells among the total number of cells (Ki-67 labelling index) was measured on three cross-sections and mean values were calculated for each group.

### Statistical analysis

All values are expressed as mean  $\pm$  SE. Statistical analysis was performed using Student's *t*-test and one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test. Analyses were conducted using SPSS 16.0 for Windows (SPSS Inc, Chicago, IL, USA).

### Results

No morbidity or mortality was observed in any of the groups. Serum total cholesterol levels ranged between 35 and 70 mg dl<sup>-1</sup> before the initiation of the 2% cholesterol diet and increased to 1000–2100 mg dl<sup>-1</sup> after 1 week ( $P < 0.01$ ). These high cholesterol levels were sustained throughout the experiment.

### Transfection efficiency

Successful transfection of ECs and SMCs was demonstrated by DAPI staining of vein grafts in group E, showing that the nuclear FITC signal was clearly stronger than that of the cell cytoplasm, thereby documenting nuclear localisation of ODN (Fig. 1).

### Functional gene blockade

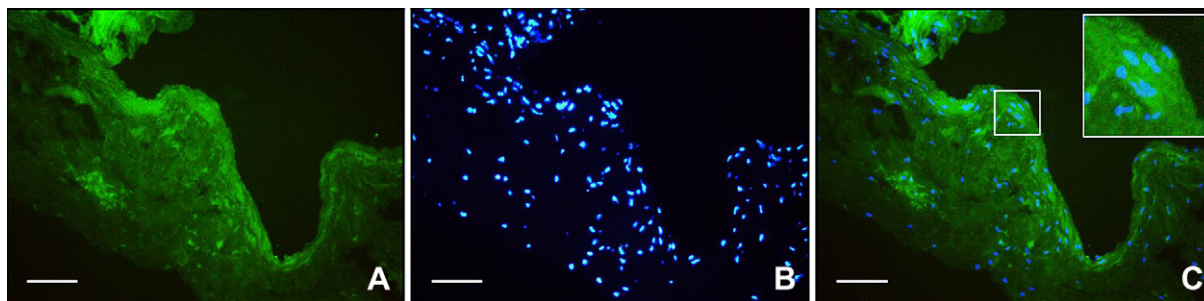
Quantitative real-time PCR of vein segments in groups B, D and E revealed that Egr-1 decoy ODN transfection achieved a 57% and 60% inhibition of Egr-1 mRNA concentration compared with vein grafts transfected with mutant decoy ODN or vein grafts that were not treated, respectively (Fig. 2).

### Inhibition of proliferation

At 6 weeks, the Ki-67 labelling index in vein grafts treated with Egr-1 decoy ODN was 44% lower compared with vein grafts treated with mutant decoy ODN, 34% lower compared with grafts treated with vehicle alone and 41% lower compared with vein grafts without any treatment (Fig. 3). The statistically significant difference was sustained at 12 weeks, with the Ki-67 labelling index in group A being 40% lower than in group B, 35% lower than in group C and 39% lower than in group D.

### Inhibition of intimal hyperplasia

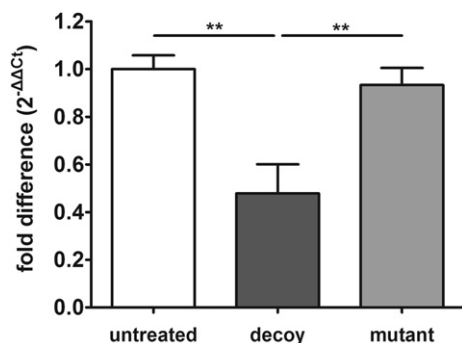
Development of eccentric atherosclerotic plaques, intimal and medial hyperplasia as well as an increase in collagen density was found in all vein grafts (Fig. 4). However, intimal thickness in the grafts treated with Egr-1 decoy ODN



**Figure 1** Nuclear localization of FITC-ODN in jugular vein graft of rabbit model. Fluorescent photomicrographs indicate concentrated green FITC-ODN signals (A), that correspond to nuclei labeled with the DAPI coloration (B), as seen in detail in the merged image (C). (Original magnification  $\times 20$ , scale bar: 200  $\mu\text{m}$ ).

( $84.3 \pm 3.3 \mu\text{m}$ , range 75.0–96.0  $\mu\text{m}$ ) was 50% lower at 6 weeks compared with the grafts that were treated with mutant decoy ODN ( $167.2 \pm 17.3 \mu\text{m}$ , range 96.0–204.1  $\mu\text{m}$ ), 42% lower compared with the grafts that were treated with vehicle alone ( $145.0 \pm 20.1 \mu\text{m}$ , range 97.0–210.0  $\mu\text{m}$ ) and 41% lower compared with the grafts that were untreated ( $144.5 \pm 18.2 \mu\text{m}$ , range 95.3–188.1  $\mu\text{m}$ ) (Fig. 5). At 12 weeks, intimal thickness in the grafts treated with Egr-1 decoy ODN ( $95.8 \pm 1.7 \mu\text{m}$ , range 92.6–97.3  $\mu\text{m}$ ) was 32% ( $145.9 \pm 11.1 \mu\text{m}$ , range 106.8–188.0  $\mu\text{m}$ ), 30% ( $142.7 \pm 21.6 \mu\text{m}$ , range 92.6–210.4  $\mu\text{m}$ ) and 36% ( $153.2 \pm 15.8 \mu\text{m}$ , range 102.5–199.5  $\mu\text{m}$ ) lower, respectively (Fig. 5). No significant difference in intimal thickness was found between vein grafts treated with mutant decoy ODN, vehicle alone or vein grafts that were untreated. No statistically significant difference was also found in overall or medial thickness between the various groups (Fig. 5).

The intimal cross-sectional area in the Egr1 decoy ODN group was 54%, 45% and 41% lower compared with the mutant ODN, vehicle alone and the untreated vein grafts respectively at 1.5 months and 39%, 35% and 40% lower, respectively, at 3 months (Fig. 6). No significant difference was found in medial thickness between the various groups. On the contrary, luminal area was significantly increased in the Egr-1 decoy ODN group, being 28%, 32% and 35% larger compared with the mutant ODN, vehicle alone and the untreated vein grafts, respectively, at 1.5 months and 39%, 29% and 44% larger, respectively, at 3 months (Fig. 7).



**Figure 2** Egr-1 mRNA expression in qRT-PCR. Egr-1 expression was 60% and 57% lower in the decoy treated grafts compared to the untreated and mutant ODN treated grafts, respectively ( $n = 6$  for each group,  $** P < 0.001$ ).

## Discussion

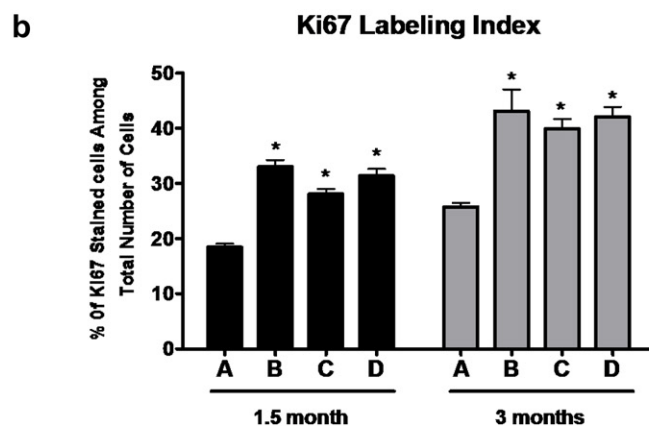
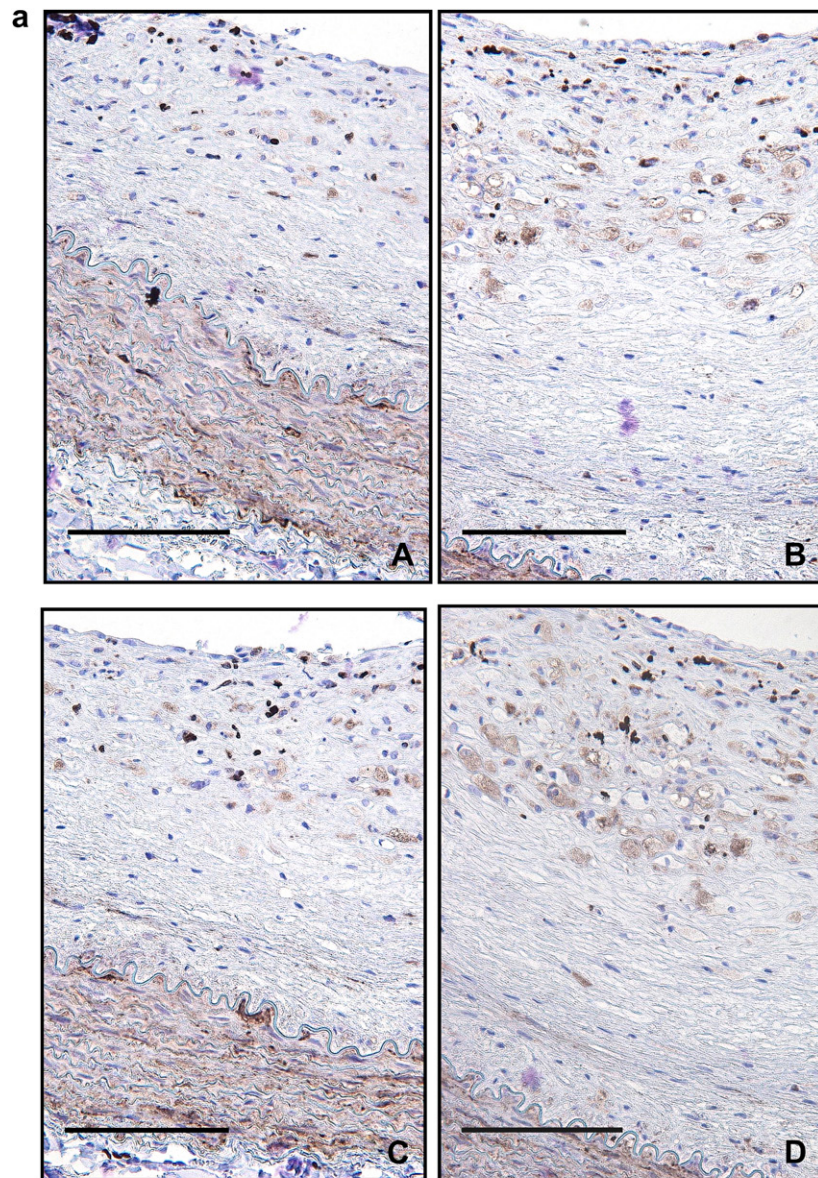
Intimal hyperplasia of vein grafts is a multifactorial process, triggered by vascular injury, altered haemodynamic stresses, vasospasm, inflammation and ischaemia. Even more complex are the intracellular pathways mediating the response of the vascular wall to these stimuli. The signalling pathways involve inhibitory proteins, such as the retinoblastoma protein, p21, p27 and p53, as well as proliferative proteins, such as cyclin-dependent kinases (cdks) and PCNA.<sup>20</sup> It is therefore clear that inhibition or promotion of a single pathway will have limited success in preventing intimal hyperplasia. In this context, transcription factors are of particular importance since they are a point of integration of multiple signalling pathways that regulate a coordinated cellular response.

Transcription factors that have been used as targets for decoy ODNs include E2F, nuclear factor-kappa B (NF $\kappa$ B), activator protein-1 (AP-1) and the CCAAT/enhancer binding protein (C/EBP). Intra-operative transfection of jugular vein to carotid artery interposition grafts with E2F decoy ODN yielded an inhibition to neointimal hyperplasia and atherosclerotic plaque formation over 6 months in hypercholesterolaemic rabbits.<sup>21</sup> In humans, E2F-decoy-treated infrainguinal vein grafts had fewer occlusions, revisions or arterial stenoses at 12 months.<sup>22</sup> On the contrary, E2F decoy proved to be no more effective than placebo in preventing angiographic graft failure, acute adverse effects, or major adverse cardiac events 12–18 months after coronary artery bypass graft (CABG).<sup>23</sup>

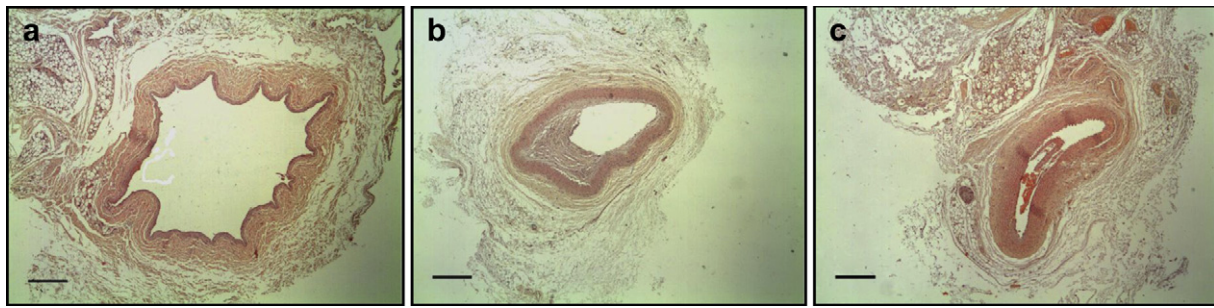
In a similar animal model, intra-operative treatment of jugular vein grafts with NF $\kappa$ B decoy ODN significantly suppressed intimal hyperplasia (by about 50%) 4 weeks after implantation, increased medial thickening and suppressed inflammatory changes and accumulation of vascular SMCs in the neointima of rabbit vein grafts.<sup>24</sup> The usefulness and safety of the NF $\kappa$ B decoy ODN transfer has also been tested in clinical practice, in 17 patients submitted to percutaneous coronary interventions (PCIs).<sup>25</sup> Six months after the PCI and decoy ODN transfection, significant restenosis was found in only one of the 17 patients. No in-stent thrombosis was found and no significant systemic adverse effect occurred in any of the patients during this observation period.

In a rabbit model of restenosis that combines balloon injury of the carotid artery with cholesterol-mediated





**Figure 3** a. Photomicrographs showing representative cross sections of vein grafts treated with Egr-1 decoy ODN (A), mutant ODN (B), vehicle alone (C) and vein grafts without any treatment (D) 1.5 month after placement in ipsilateral carotid artery (immunohistochemical staining for Ki-67, original magnification  $\times 20$ , scale bar: 200  $\mu\text{m}$ ). b. Inhibitory effect of Egr-1 decoy transfection on proliferation (Ki-67 positive cells) at 1.5 and 3 months after placement of vein grafts treated with Egr-1 decoy ODN (A), mutant ODN (B), vehicle alone (C) and vein grafts without any treatment (D) ( $n = 6$  for each group,  $* P < 0.05$ ).



**Figure 4** Light micrographs of normal vein (a), Egr-1 decoy ODN-treated vein grafts (b) and mutant decoy ODN-treated vein grafts (c) in hypercholesterolemic rabbits 3 months after grafting. An intimal and medial hyperplasia is seen in the vein grafts compared to the normal vein (hematoxylin-eosin stain: original magnification  $\times 5$ , scale bar: 500  $\mu\text{m}$ ).

chronic inflammation, a decoy ODN capable of neutralising C/EBP achieved a significant reduction (up to 50%) of neo-intimal formation and intravascular inflammation.<sup>26</sup> Other authors have used the haemagglutinating virus of Japan (HVJ)-liposome technique to transfect AP-1 decoy ODN into rat carotid arteries.<sup>27,28</sup> The AP-1 decoy ODN, introduced in this way, significantly reduced neointimal formation after balloon injury to the rat carotid artery.

The quest of the ideal target to treat bypass failure is stronger than ever, though one might argue that the ideal target exists. The dream of the discovery of a single transcription factor, the binding of which would be enough by itself to prevent intimal hyperplasia might prove elusive. Our study adds another transcription factor, Egr-1, the binding of which with an ODN achieved a smaller but still statistically significant suppression of intimal hyperplasia by about 40% at 6 weeks and 30% at 3 months.

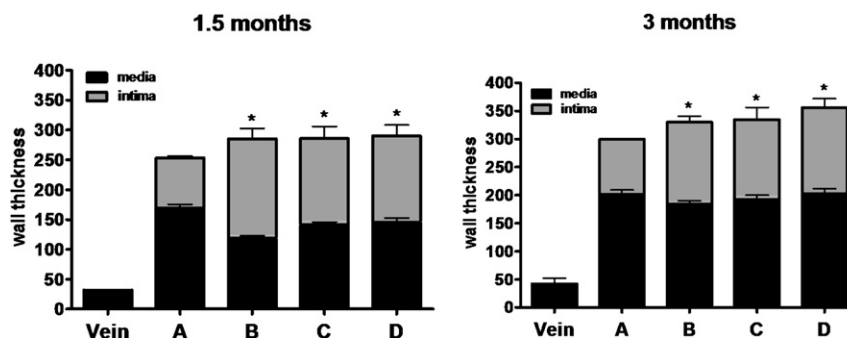
Prompted by the finding that multiple processes participate in the formation of neointimal hyperplasia, Miyake et al. developed a chimeric decoy ODN approach, assessing the effect of simultaneous inhibition of NF $\kappa$ B and E2F on the formation of neointimal hyperplasia.<sup>29</sup> A 2-cm diameter polytetrafluoroethylene (PTFE) graft was placed in the carotid artery in an end-to-end fashion in hypercholesterolaemic rabbits and chimeric decoy ODNs were introduced in both ends of the carotid artery using pressure-mediated transfection. Treatment with chimeric decoy ODN significantly inhibited

proximal and distal anastomotic intimal hyperplasia, SMC proliferation and macrophage accumulation, whereas it accelerated re-endothelialisation.

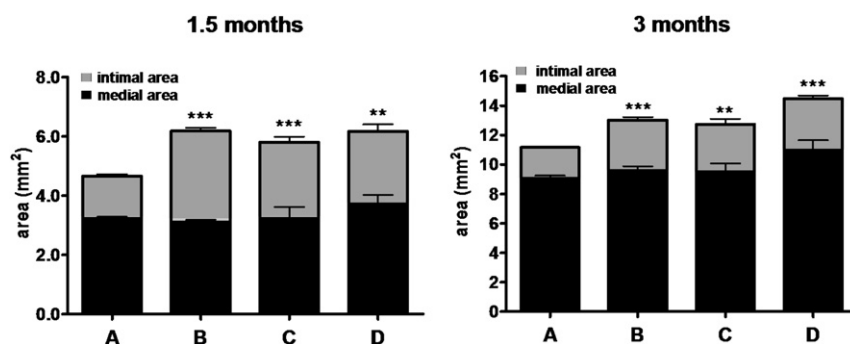
The reason why most relevant studies, including ours, have used a hypercholesterolaemic diet,<sup>18,21,24,29</sup> is that the rabbit hypercholesterolaemic model is considered as the most relevant and appropriate for studying novel approaches against human restenosis.<sup>18</sup> That is because hypercholesterolaemia has been shown to induce accelerated development of intimal hyperplasia in experimental vein grafts.<sup>30</sup>

A critical point in all transfection studies is the efficiency of the transfection. Gene transfer methods can be roughly divided into two major categories, viral and non-viral. The most commonly used non-viral approaches are direct incubation with naked DNA and coupling of DNA with lipophilic/hydrophobic agents.

The use of naked DNA is simple and safe but carries the risk of extracellular nuclease degradation. Coupling the DNA with cationic phospholipids improves transfection efficiency but may induce inflammation.<sup>31</sup> Viral vectors, on the other hand, produce a higher efficiency of gene transfer but pose potential hazards of triggering an immune response to the viral proteins. In our study, successful delivery of the ODN into the nuclei of the target cells was verified by DAPI staining, while inhibition of target gene expression was confirmed by RT-PCR. The resulting 60%



**Figure 5** Intimal and medial wall thickness at 1.5 and 3 months after placement of vein grafts treated with Egr-1 decoy ODN (A), mutant ODN (B), vehicle alone (C) and vein grafts without any treatment (D) in cholesterol-fed rabbits. Intimal hyperplasia of Egr1 decoy ODN versus mutant ODN, vehicle alone and the untreated vein grafts was 50%, 42% and 41% reduced respectively at 1.5 months and 32%, 30% and 36% reduced respectively at 3 months ( $n = 6$  for each group,  $* P < 0.05$ ). Gray bars: intimal thickness ( $\mu\text{m}$ ), black bars: medial thickness ( $\mu\text{m}$ ).



**Figure 6** Intimal and medial cross-sectional wall area at 1.5 and 3 months after placement of vein grafts treated with Egr-1 decoy ODN (A), mutant ODN (B), vehicle alone (C) and vein grafts without any treatment (D) in cholesterol-fed rabbits. The intimal area of Egr1 decoy ODN versus mutant ODN, vehicle alone and the untreated vein grafts was 54%, 45% and 41% reduced respectively at 1.5 months and 39%, 35% and 40% reduced respectively at 3 months ( $n = 6$  for each group, \*\*  $P < 0.001$ , \*\*\*  $P < 0.0001$ ). Gray bars: intimal area (mm<sup>2</sup>), black bars: medial area (mm<sup>2</sup>).

decrease in Egr-1 mRNA concentration is quite satisfactory and proved to be enough to achieve a therapeutic effect.

The major drawback of antisense ODNs is their relatively fast nucleolytic degradation. The superior stability of short interfering RNA (siRNA) makes it a promising strategy of gene inhibition and is currently studied in different disease models. In a recently published study, local lentiviral short hairpin RNA silencing of CCR2 reduced vein graft thickening in hypercholesterolaemic apolipoprotein E3-Leiden mice by 38%, 4 weeks after surgery.<sup>32</sup>

A theoretical question that might be raised is how an intra-operative vein graft transfection could have a prolonged or, ideally, permanent beneficial effect on the vein wall. The answer is that target gene inhibition does not necessarily need to be permanent. During the first few weeks after implantation, the vein graft recovers from the acute traumatic, ischaemic and inflammatory injuries associated with grafting and becomes less susceptible to

neointimal hyperplasia and accelerated atherosclerosis. In any case, of course, the strategy with the more permanent effect would probably be more beneficial, combating not only the acute response to the new haemodynamic environment but also the effect of other patient-related risk factors, such as smoking or hypercholesterolaemia. The use of techniques with a more permanent effect (such as siRNA), chimeric decoy ODNs (targeting more than one transcription factors) and optimised introduction methods may revive the interest in decoy ODN clinical research which was diminished after the failure of E2F decoy to inhibit restenosis in coronary artery bypass grafts.

In conclusion, ODN delivery in vein grafts interposed in the carotid arteries was achieved by only increasing the hydrostatic pressure and without using a specific vehicle. Inhibition of Egr-1 expression seems to contribute in the significant reduction in the development of neointimal hyperplasia. These results are encouraging in the context of decelerating neointimal hyperplasia progression, while the proposed method is technically simple and efficient, low cost and not requiring any specific operational expertise.

## Conflict of Interest

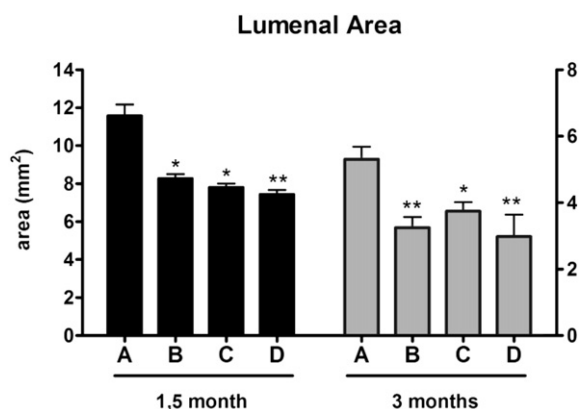
None.

## Funding

This research project has been implemented within the framework of the 'Kapodistrias' programme, financed by the Special Account for Research Grants of the National and Kapodistrian University of Athens.

## References

- 1 Carrel AGC. Uniterminal and biterminal venous transplantations. *Surg Gynecol Obstet*; 1906:266–86.
- 2 Neale ML, Graham JC, Lane RJ, Cheung DS, Appleberg M. The influence of graft type on patency of infrainguinal arterial bypass grafts. *J Am Coll Surg* 1994;178:155–63.
- 3 Donaldson MC, Mannick JA, Whittemore AD. Causes of primary graft failure after in situ saphenous vein bypass grafting. *J Vasc Surg* 1992;15:113–8.



**Figure 7** Effect of Egr-1 decoy transfection in luminal area 1.5 and 3 months after placement of vein grafts treated with Egr-1 decoy ODN (A), mutant ODN (B), vehicle alone (C) and vein grafts without any treatment (D) in cholesterol-fed rabbits. The lumen area of Egr-1 decoy ODN versus scrambled ODN, vehicle alone and the untreated vein grafts was 28%, 32% and 35% increased respectively at 1.5 months and 39%, 29% and 44% increased respectively at 3 months ( $n = 6$  for each group, \*  $P < 0.05$ , \*\*  $P < 0.001$ ).



- 4 Sarkar S, Salacinski HJ, Hamilton G, Seifalian AM. The mechanical properties of infrainguinal vascular bypass grafts: their role in influencing patency. *Eur J Vasc Endovasc Surg* 2006;**31**:627–36.
- 5 Corpataux JM, Naik J, Porter KE, London NJ. A comparison of six statins on the development of intimal hyperplasia in a human vein culture model. *Eur J Vasc Endovasc Surg* 2005;**29**:177–81.
- 6 Schachner T, Zou Y, Oberhuber A, Tzankov A, Mairinger T, Laufer G, et al. Local application of rapamycin inhibits neo-intimal hyperplasia in experimental vein grafts. *Ann Thorac Surg* 2004;**77**:1580–5.
- 7 Jeremy JY, Gadsdon P, Shukla N, Vijayan V, Wyatt M, Newby AC, et al. On the biology of saphenous vein grafts fitted with external synthetic sheaths and stents. *Biomaterials* 2007;**28**:895–908.
- 8 Ulus AT, Tütün U, Zorlu F, Can C, Apaydin N, Karacagil S, et al. Prevention of intimal hyperplasia by single-dose pre-insertion external radiation in canine-vein interposition grafts. *Eur J Vasc Endovasc Surg* 2000;**19**:456–60.
- 9 Trerotola SO, Carmody TJ, Timmerman RD, Bergan KA, Dreesen RG, Frost SV, et al. Brachytherapy for the prevention of stenosis in a canine hemodialysis graft model: preliminary observations. *Radiology* 1999;**212**:748–54.
- 10 Yamaguchi A, Woodburn KW, Hayase M, Robbins RC. Reduction of vein graft disease using photodynamic therapy with motexafin lutetium in a rodent isograft model. *Circulation* 2000;**102**:III275–III280.
- 11 Wallitt EJ, Jevon M, Hornick PI. Therapeutics of vein graft intimal hyperplasia: 100 years on. *Ann Thorac Surg* 2007;**84**:317–23.
- 12 Schachner T. Pharmacologic inhibition of vein graft neointimal hyperplasia. *J Thorac Cardiovasc Surg* 2006;**131**:1065–72.
- 13 Bhardwaj S, Roy H, Ylä-Herttua S. Gene therapy to prevent occlusion of venous bypass grafts. *Expert Rev Cardiovasc Ther* 2008;**6**:641–52.
- 14 Sukhatme VP, Cao XM, Chang LC, Tsai-Morris CH, Stamenkovich D, Ferreira PC, et al. A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. *Cell* 1988;**53**:37–43.
- 15 Silverman ES, Collins T. Pathways of Egr-1-mediated gene transcription in vascular biology. *Am J Pathol* 1999;**154**:665–70.
- 16 Blaschke F, Bruemmer D, Law RE. Egr-1 is a major vascular pathogenic transcription factor in atherosclerosis and restenosis. *Rev Endocr Metab Disord* 2004;**5**:249–54.
- 17 McCaffrey TA, Fu C, Du B, Eksinar S, Kent KC, Bush Jr H, et al. High-level expression of Egr-1 and Egr-1-inducible genes in mouse and human atherosclerosis. *J Clin Invest* 2000;**105**:653–62.
- 18 Ohtani K, Egashira K, Usui M, Ishibashi M, Hiasa KI, Zhao Q, et al. Inhibition of neointimal hyperplasia after balloon injury by cis-element 'decoy' of early growth response gene-1 in hypercholesterolemic rabbits. *Gene Ther* 2004;**11**:126–32.
- 19 Livak KJ, Schmittgen TB. Analysis of relative gene expression data using real time quantitative PCR and the  $2^{-\Delta\Delta C_T}$  method. *Methods* 2001;**25**:402–8.
- 20 Hoel AW, Conte MS. Edifoligide: a transcription factor decoy to modulate smooth muscle cell proliferation in vein bypass. *Cardiovasc Drug Rev* 2007;**25**:221–34.
- 21 Ehsan A, Dell'Acqua MDG, Dzau VJ. Long-term stabilization of vein graft wall architecture and prolonged resistance to experimental atherosclerosis after e2f decoy oligonucleotide gene therapy. *J Thor Cardiovasc Sur* 2001;**121**:714–22.
- 22 Mann MJ, Whittemore AD, Donaldson MC, Belkin M, Conte MS, Polak JF, et al. Ex-vivo gene therapy of human vascular bypass grafts with E2F decoy: the prevent single-centre, randomised, controlled trial. *Lancet* 1999;**354**:1493–8.
- 23 Alexander JH, Hafley G, Harrington RA, Peterson ED, Ferguson Jr TB, Lorenz TJ, et al. PREVENT IV Investigators. Efficacy and safety of edifoligide, an E2F transcription factor decoy, for prevention of vein graft failure following coronary artery bypass graft surgery: PREVENT IV: a randomized controlled trial. *JAMA* 2005;**294**:2446–54.
- 24 Miyake T, Aoki M, Shiraya S, Tanemoto K, Ogihara T, Kaneda Y, et al. Inhibitory effects of NFkappaB decoy oligodeoxynucleotides on neointimal hyperplasia in a rabbit vein graft model. *J Mol Cell Cardiol* 2006;**41**:431–40.
- 25 Egashira K, Suzuki J, Ito H, Aoki M, Isobe M, Morishita R. Long-term follow up of initial clinical cases with NF-kappaB decoy oligodeoxynucleotide transfection at the site of coronary stenting. *J Gene Med* 2008;**10**:805–9.
- 26 Kelkenberg U, Wagner AH, Sarhaddar J, Hecker M, von der Leyen HE. CCAAT/enhancer-binding protein decoy oligodeoxynucleotide inhibition of macrophage-rich vascular lesion formation in hypercholesterolemic rabbits. *Arterioscler Thromb Vasc Biol* 2002;**22**:949–54.
- 27 Ahn JD, Morishita R, Kaneda Y, Lee SJ, Kwon KY, Choi SY, et al. Inhibitory effects of novel AP-1 decoy oligodeoxynucleotides on vascular smooth muscle cell proliferation in vitro and neointimal formation in vivo. *Circ Res* 2002;**90**:1325–32.
- 28 Kume M, Komori K, Matsumoto T, Onohara T, Takeuchi K, Yonemitsu Y, et al. Administration of a decoy against the activator protein-1 binding site suppresses neointimal thickening in rabbit balloon-injured arteries. *Circulation* 2002;**105**:1226–32.
- 29 Miyake T, Aoki M, Morishita R. Inhibition of anastomotic intimal hyperplasia using a chimeric decoy strategy against NFkappaB and E2F in a rabbit model. *Cardiovasc Res* 2008;**79**:706–14.
- 30 Klyachkin ML, Davies MG, Svendsen E, Kim JH, Massey MF, Barber L, et al. Hypercholesterolemia and experimental vein grafts: accelerated development of intimal hyperplasia and an increase in abnormal vasomotor function. *J Surg Res* 1993;**54**:451–68.
- 31 Norman J, Denham W, Denham D, Yang J, Carter G, Abouhamze A, et al. Liposome-mediated, nonviral gene transfer induces a systemic inflammatory response which can exacerbate pre-existing inflammation. *Gene Therapy* 2000;**7**:1425–30.
- 32 Eefting D, Bot I, de Vries MR, Schepers A, van Bockel JH, Van Berkel TJ, et al. Local lentiviral short hairpin RNA silencing of CCR2 inhibits vein graft thickening in hypercholesterolemic apolipoprotein E3-Leiden mice. *J Vasc Surg* 2009;**50**:152–60.